Effectiveness of AFLPs and Retrotransposon-Based Markers for the Identification of Portuguese Grapevine Cultivars and Clones

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Abstract Grapevine germplasm, including 38 of the main Portuguese cultivars and three foreign cultivars, Pinot Noir, Pinot Blanc and Chasselas, used as a reference, and 37 true-to-type clones from the Alvarinho, Arinto, Loureiro, Moscatel Galego Branco, Trajadura and Vinhão cultivars were studied using AFLP and three retrotransposon-based molecular techniques, IRAP, REMAP and SSAP. To study the retrotransposon-based polymorphisms, 18 primers based on the LTR sequences of *Tvv1*, *Gret1* and *Vine-1* were used. In the analysis of 41 cultivars, 517 IRAP, REMAP, AFLP and SSAP fragments were obtained, 83% of which were polymorphic. For IRAP, only the *Tvv1*Fa primer amplified DNA fragments. In the REMAP analysis, the *Tvv1*Fa-Ms14 primer combination only produced polymorphic bands, and the *Vine-1* primers produced mainly ISSR fragments. The highest number of polymorphic fragments was found for AFLP. Both AFLP and SSAP showed a greater capacity for identifying clones, resulting in 15 and 9 clones identified, respectively. Together, all of the techniques allowed for the identification of 54% of the studied clones, which is an important step in solving one of the challenges that viticulture currently faces.

Keywords AFLP · IRAP · REMAP · SSAP · *Vitis vinifera* L. · Clonal identification

Introduction

Until recently, ampelography was the only tool available for the identification of grapevine cultivars and clones; however, this technique is time consuming, often subjective and complex, and it can be blurred by environmental factors, such as climate, pathogens, soil characteristics, or plant-nutritional status. Isoenzymes, and more recently, restriction fragment length polymorphisms (RFLPs), were the first non-morphological markers to be applied to identifying grapevine and other species. The discovery of PCR opened up the field of marker design to include a broad range of molecular markers, each one with their own set of advantages and disadvantages. Although microsatellites (or simple sequence repeats, SSRs) are the markers of choice for grapevine cultivars identification, they are monolocus, and the number of loci that can be amplified in one PCR reaction is limited. In contrast, multilocus molecular markers are potentially more useful for analysing clustering through the use of genetic relationships between cultivars. Moreover, although the recent identification of some SSR loci that appear to be suitable for clonal identification [1], many of them exhibit several limitations.

Amplified fragment length polymorphisms (AFLPs) have been used successfully for the analysis of grapevine clonal variation [2–5]. This methodology combines the capabilities of RFLPs with the flexibility of PCR-based technology by linking primer recognition sequences (adaptors) to the restricted DNA and the selective PCR amplification of restriction fragments through the use of a

limited set of primers. The AFLP technique generates fingerprints of any DNA, regardless of its source, has the advantage of not requiring prior knowledge of the DNA sequence [6], and it is a firmly established molecular marker technique that is considered to be highly reproducible and useful in plant fingerprinting [7].

Transposable elements (TEs) are ubiquitous components of all living organisms [8]. TEs are classified based on their structural features and are divided in two main classes depending on if their transposition intermediate is RNA (class I) or DNA (class II). Class I TEs, or retrotransposons, play a major role in plant gene and genome evolution and are the most abundant and widespread of the eukaryotic TE classes; in many cases, they represent over 50% of the nuclear DNA content [9]. The grapevine genome-sequencing project revealed that TEs represent approximately 41.4% [10] and that retrotransposons represent 17%–24% of the whole grapevine genome [10, 11].

Retrotransposons fall into clearly separate groups, long terminal repeat (LTR)-containing elements, elements that lack LTRs, long-interspersed elements (LINEs) and short-interspersed elements (SINEs) [12]. LTR retrotransposons are sub-classified into two major families: the Ty1-*copia* and the Ty3-*gypsy* groups. *Gret1* (Grape retrotransposon 1), which was the first complete retrotransposon sequence to be isolated from a *Vitis* genome, is a *gypsy*-like LTR retrotransposon that is a retrotransposon-induced mutation of the *VvmybA1* gene, which affects the colour of the grape skin [13]. Before the identification of *Gret1*, only two partial sequences of two *copia*-like retrotransposons had been isolated from grapevine: *Vine-1* (*Vitis vinifera* Element-1), a retrotransposon that is inserted into the fourth exon of the *Adhr* gene in various grapevine cultivars [14], and a family of retroelements named *Tvv1* [15]. Both *Gret1* and *Vine-1* were isolated from the genomic regions directly upstream from or inside coding sequences. Therefore, retrotransposons play a significant role in gene expression and in the phenotypic variation of grapevine, and several of them affect important agronomical traits [16].

The ubiquitous nature of retrotransposons and their involvement in creating genomic diversity by stably integrating large DNA segments into dispersed chromosomal loci make retrotransposons ideal for developing as molecular markers. Integration sites that are shared between germplasm accessions have a high probability of being present in their last common ancestor. Therefore, retrotransposon insertion polymorphisms can help one to establish pedigrees and phylogenies and can serve as biodiversity indicators [12]. In recent years, several molecular marker methods based on retrotransposons have been developed, which rely on the principle that a joint is formed between the retrotransposon and the genomic DNA during the integration process.

The sequence-specific amplified polymorphism (SSAP) method [17] is a derivation of AFLPs, as it amplifies products between a retrotransposon integration site and a restriction site to which an adapter has been linked. The inter-retrotransposon amplified polymorphism (IRAP) generates PCR products between two nearby retrotransposons using two identical or different primers inserted sufficiently close to each other to allow for efficient amplification, and primers matching the outer segments of the LTRs are generally utilised [18].

Retrotransposon-microsatellite-amplified polymorphism (REMAP) uses one LTR primer and a primer designed for annealing to the 3′ end of a stretch of SSR, and it detects retrotransposons that are integrated near to SSR sequences [18].

The retrotransposon-based markers have been applied successfully to the analysis of genetic relationships, phylogenetic evolution and genetic diversity in genera and species as diverse as persimmon [19], apple [15, 20], flax [21], sunflower [22], pea [23] and wheat [24]. Moreover, these molecular markers are used in the construction of genetic maps and the identification of genes, providing insights into plant evolution [25]. In grapevine, the use of retrotransposons to study polymorphisms at the genus level in *Vitis* [26] and at the cultivars and clones level, in *Vitis vinifera* L., has shown promising results. Relying on the presence of retrotransposon reverse transcriptase sequences, inverse sequence-tagged repeat (ISTR) analyses have been conducted to investigate the genetic diversity amongst the closely related Sangiovese accessions and have identified a high level of polymorphism [27]. SSAP analyses, by implementing the primers based on the LTRs of *Vine-1*, distinguished clones of the Traminer cultivar, but not of the Pinot cultivar [28]. In a study of the Portuguese cultivars Castelão, Cerceal Branco, Loureiro and Touriga Nacional (the last two are also included in this study), REMAP and IRAP molecular markers based on the LTRs of *Gret1* revealed the existence of polymorphism between cultivars, whereas the markers failed to detect polymorphism between clones [29]. Pelsy et al. [30] assessed the discriminating power of SSAPs, which relied on the LTRs of grapevine retrotransposons, and confirmed the efficiency of the SSAPs in distinguishing between 12 grapevine varieties. High percentages of polymorphism have been identified in Malbec and Syrah clones through SSAP analysis using primers that are based on the LTRs of *Vine-1* [4].

Portuguese viticulture is considered to be rich in varietal biodiversity. In the beginning of the twentieth century, approximately 100 different cultivars were recorded in certain municipalities, and more than 900 were recorded in the country as a whole. Although these numbers likely include several duplications, they indicate the existence of a high biodiversity. At present, the list of Portuguese

grapevine cultivars that are suitable for wine production consists of 341 names; some of these cultivars are present only in germplasm collections, and still others are not included in this list [31].

Due to existing legislation, there has been a decrease in recent years in the number of cultivars that are authorised for the production of quality wine, resulting in the risk of extinction of some ancient and minor varieties. A group of less than 10 cultivars, including white and black, represent approximately 80% of the Portuguese grapevine area [32]. Moreover, some international cultivars have had a great impact on the viticulture of the country.

Polymorphism between clones is mainly the result of somatic mutations, and it causes genetic diversity, such as point mutations, large deletions, illegitimate recombination or variable numbers of repeats in microsatellite sequences [33]. Portugal began its grapevine clonal selection program in 1978 to preserve its existing variability. Subsequent to a nationwide survey, this program concentrated on 51 cultivars, and certified clones were selected and preserved for propagation, for breeding programs or for studies of their origins.

The present study compares the effectiveness of AFLP and three retrotransposon-based molecular techniques, IRAP, REMAP and SSAP, for the identification of cultivars and clones that include 38 Portuguese and 3 foreign cultivars and 37 clones.

Materials and Methods

Plant Material and DNA Isolation

Table 1 shows the 41 grapevine cultivars and 37 clones, of 6 of them, that were analysed. The clones belong to important cultivars from the 'Vinhos Verdes' Portuguese Controlled Designation of Origin (DOC): Alvarinho, Loureiro, Vinhão, Trajadura, Arinto, and the 'Douro' DOC region Moscatel Galego Branco, a synonym for the Muscat à Petit Grains that is used in the production of the highly appreciated licorous wine 'Moscatel de Favaios'.

DNA was extracted from young leaves as per the procedure described by Mulcahy et al. [34] and modified by Vignani et al. [35]. The cultivar identity was previously confirmed using 12 SSR loci, including the six nuclear microsatellite loci OIV 801–806 [36] and six other loci [31].

Primer Design

In total, 18 primers were designed based on the LTR sequences of *Vine-1* (AF116598 [14]), *Tvv1* (AF478364 to AF478390 [15]) and *Gret1* (AB111100 [13]) RTs. Of the 18 primers, 11 were used previously by D'Onofrio et al. [26], and 7 were designed in this study as indicated by those authors (Table 2).

A total of 20 ISSR primers containing two or three nucleotide repeats and, in some cases, one selective nucleotide at the 3′-end were used (Table 3) [26]. For the three UBC primers (University of British Columbia, Vancouver, Canada) (UBC-888, UBC-889, and UBC-890), two additional nucleotides were added to the 3′-end, and also the UBC-888 primer was designed as the reverse of the original; the modifications were performed to reduce the amplification of ISSR fragments.

IRAP Amplification

IRAP analysis was performed as per the method described by Kalendar et al. [18]. Forward and reverse LTR primers were designed using each retrotransposon family (*Tvv1*, *Gret1* and *Vine-1*—see Table 2) and were tested alone or in combination. PCR reactions and resolution of the PCR products were performed according to methods previously described [26].

REMAP Amplification

For the REMAP analysis, each LTR primer was used in combination with 3′- or 5′-anchored ISSR primers (Tables 2, 3). The PCR reactions and PCR product resolutions were performed as described for IRAP. For IRAP and REMAP analysis, a set of experiments was performed to identify the most polymorphic LTR primer for each retrotransposon.

The following precautions were taken in the REMAP amplifications to ensure that ISSR and inter-retrotransposon fragments were not considered: a group of cultivars were amplified only with an ISSR primer, only with a LTR primer or with both primers, and the ISSR or inter-retrotransposon amplifications were discarded.

AFLP Amplification

AFLP analysis was carried out using methods previously described [2]. DNA was digested with *Eco*RI and *Mse*I and was ligated to *Eco*RI and *Mse*I adapters. For selective amplification, a 5′-end 6-FAM fluorophore-labelled E34 primer that is complementary to *Eco*RI and that has three additional selective nucleotides was used in combination with M36 and M40 primers, which are complementary to *Mse*I and have three additional selective nucleotides [28]. The amplified products were run on an ABI Prism® 3730 Genetic Analyzer using the GeneScan™ 500 LIZ® size standard (PE Applied Biosystems, Foster City, CA, USA). Labelled products were analysed and sized using Peak Scanner™ v1.0 free software (PE Applied Biosystems, Foster City, CA, USA).

Table 1 List of the Portuguese grapevine cultivars and clones used in this study as well as three foreign varieties (*in italics*) that were used for reference

Cultivar	Berry colour	Clones
Alfrocheiro	B	
Alvarelhão	B	
Alvarinho	W	AL0122, AL1011, **AL1025**, **AL1050**
Amaral	B	
Aragonez	B	
Arinto	W	**AR2404**, **AR2425**, **AR3502**, AR7503, **AR8204**
Arinto do Interior	W	
Avesso	W	
Azal	W	
Baga	B	
Barca	B	
Batoca	W	
Bical	W	
Borraçal	B	
Espadeiro	B	
Gouveio	W	
Lameiro	W	
Loureiro	W	L0419, L0719, **L0734**, **L0959**, L1105
Malvasia Fina	W	
Melhorio	B	
Moscatel Galego Branco	W	**MG0207**, **MG0420**, **MG0501**, MG0508, MG0514, MG0524, MG0538, MG0809, MG1104, **MG1118**, MG1302, **MG1306**
Moscatel Galego Roxo	R	
Padeiro	B	
Pedral	B	
Rabigato	W	
Rabo de Ovelha	W	
Sousão	B	
Sousão Galego	B	
Tália	W	
Tinta Barroca	B	
Tinta Carvalha	B	
Tinta Francisca	B	
Tinto Cão	B	
Touriga Franca	B	
Touriga Nacional	B	
Trajadura	W	TJ0332, **TJ1020**, **TJ1103**, **TJ1126**, **TJ1207**
Trincadeira	B	
Vinhão	B	VN0249, **VN0340**, **VN0514**, **VN0530**, VN1913, VN2143
Chasselas	W	
Pinot Noir	B	
Pinot Blanc	W	

Clones that were identified are shown in *bold*

W white, *B* black, *R* red or pink

SSAP Amplification

The SSAP analysis was performed following the same protocol as outlined for AFLP, but instead of the E34 primer, 5′-end 6-FAM fluorophore-labelled *Tvv1*Fa and *Gret1*Fa primers were used in combination with the M36 primer. The SSAP products were separated using an ABI Prism® 3730 Genetic Analyzer following methods previously described [26].

Data Scoring and Analysis

DNA and technical replicates were included in the analysis and demonstrate the reproducibility of each molecular marker.

Binary matrices (presence/absence) were prepared from AFLP and SSAP products that were analysed and sized using Peak Scanner™ Software, and from IRAP and REMAP electrophoretic band patterns. Each PCR product was assumed to represent a single locus. The Shannon information index (SI), the marker index (MI) and the polymorphic information content (PIC) were calculated.

In general, for a specific locus in a given sample, the SI was calculated as follows: $-\sum p_i \log_2 p_i$, where p_i is the allelic frequency of the ith allele in question for the specific sample, and the calculation was performed using the PopGene software version 1.32 [37].

The MI was calculated as the product of two functions—the PIC and the effective multiplex ratio (EMR). For dominant markers, PIC $= 1 - [f^2 + (1 - f)^2]$, where f is the frequency of the marker in the dataset, with the maximum PIC 0.5 for $f = 0.5$ [38]. The EMR of a primer combination is defined as $E = nP_{0.95}$, where n is the number of loci detected per primer combination, and $P_{0.95}$ is the percentage of polymorphic loci at 95% criterion [39]. $P_{0.95}$ was calculated using the TFPGA 1.3 software [40].

AFLP, SSAP, IRAP and REMAP binary matrices were imported into the NTSYS-pc 2.02g package [41] for cluster analysis. Genetic similarity matrices among genotypes were calculated according to the simple matching (SM) similarity index [42] using the SIMQUAL routine. Dendrograms were built based on similarity coefficients using the unweighted

Table 2 Primers designed using LTR sequences from *Gret1*, *Tvv1* and *Vine-1* grapevine retrotransposons (RT)

RT	LTR primer	Primer orientation	Primer sequence (5′–3′)	Reference
Gret1	*Gret1*Fa	Forward	(AG)TGCGTCC(AG)GACACCCTGT	[26]
	*Gret1*Fb	Forward	GGTAAAAGGCCAACT(AG)ATTTAT	[26]
	*Gret1*Fc	Forward	CCATGGCTAACAAAACCATC	This study
	*Gret1*PPT	Forward	CAAAATAGGCAAGTTAAAAAGG	This study
	*Gret1*Ra	Reverse	CTTAAGGGAATCCGGATGAT	[26]
	*Gret1*Rb	Reverse	TATGAAGGTTGTCCGGATGT	[26]
Tvv1	*Tvv1*Fa	Forward	TCCA(AG)CTTCAGGGGGAGTGT	[26]
	*Tvv1*Fb	Forward	CCATAATTT(GC)GTT(GC)TTTCCTTA	[26]
	*Tvv1*Fc	Forward	CCTTAGGGATAATACCTTCCTAATTT	This study
	Pltr1	Forward	CCTAATTCAGGACTCTCAAT	This stydy
	*Tvv1*Ra	Reverse	TATATATACAATTGAGAGTCCTAA	[26]
	P17	Reverse	(CT)AGAATTCTTACTCTCTTCC	This study
Vine-1	*Vine1*Fa	Forward	TTCAGCACTCTTCATCAATAAA	[26]
	*Vine1*Fb	Forward	GCTTGATTTTCTGTCATTCTAA	[26]
	*Vine1*Fc	Forward	TGCAGCTGGAGTCTTACACA	This study
	*Vine1*PPT	Forward	ACGAAGTCAAGGAGGAGTGTT	This study
	*Vine1*Ra	Reverse	TATATTTTTGGGCTGCTGGA	[26]
	*Vine1*Rb	Reverse	GAAACCAAATCTTGACCAAA	[26]

Table 3 ISSR primers used in this study

ISSR primer	Primer orientation	Primer sequence (5′–3′)	Reference[a]
Ms1	Forward	TCCTCCTCCTCCTCCTCCA	[26]
Ms2	Forward	TGGTGGTGGTGGTGGTGGTGGA	[26]
Ms3	Forward	CACCACCACCACCACCACCACT	[26]
Ms4	Forward	CACACACACACACACACACAG	[26]
Ms5	Forward	GAGAGAGAGAGAGAGAGAC	[26]
Ms6	Forward	TCTCTCTCTCTCTCTCTCTCG	[26]
Ms7	Forward	AGAGAGAGAGAGAGAGAGAGG	[26]
Ms8	Forward	AGAGAGAGAGAGAGAGAGAGC	[26]
Ms9	Forward	ACACACACACACACACACACG	[26]
Ms10	Forward	ACACACACACACACACACACC	[26]
Ms11	Forward	CTCTCTCTCTCTCTCTCTCTG	[26]
Ms12	Forward	TCTCTCTCTCTCTCTCTCTCC	[26]
Ms13	Forward	GTGTGTGTGTGTGTGTGTGTC	[26]
Ms14	Forward	TGTGTGTGTGTGTGTGTGTGC	[26]
Ms15	Forward	TGTGTGTGTGTGTGTGTGTGG	[26]
UBC888	Forward	(GCT)(AGT)(GCT)CACACACACACACA	UBC
UBC888-2	Forward	(GCT)(AGT)(GCT)CACACACACACACACA	UBC + this study
UBC888-rev-2	Reverse	ACACACACACACACAC(GCT)(AGT)(GCT)	UBC + this study
UBC889-2	Forward	(AGT)(GCT)(AGT)ACACACACACACACAC	UBC + this study
UBC890-2	Forward	(AGC)(ACT)(AGC)GTGTGTGTGTGTGTGT	UBC + this study

[a] UBC University of British Columbia

pair-group method arithmetic average (UPGMA) with the NTSYS-pc 2.02g software package [41].

Bootstrap analysis (1,000 replacements) was adopted to verify that the number of polymorphic loci that were evaluated was high enough to provide accurate estimations of genetic distance. The bootstrap values were calculated using WinBoot software [43], and differences amongst the dendrograms were assessed based on correlations between

similarity matrices and between cophenetic matrices that were calculated by the Mantel matrix correspondence test [44].

Results

Cultivar Polymorphisms and Identification

Table 4 shows the selected primer combinations that were used to carry out the analysis on cultivars. Out of the 517 IRAP, REMAP, AFLP and SSAP fragments that were obtained from the 41 cultivars that were studied, 429 (83%) were polymorphic (Table 4). For IRAP, only the *Tvv1*Fa primer amplified DNA fragments. In the REMAP analysis, the *Tvv1*Fa-Ms14 primer combination only produced polymorphic bands, and the *Tvv1*Fa-UBC890-2 combination provided the highest number (28) of polymorphic bands (Table 4; Fig. 1). For SSAP, the highest number of polymorphic bands (78) was obtained using the *Gret1*Fa-M36 combination, whereas for AFLP, the E34-M36 primer combination was the most polymorphic, producing 90 (79.6%) polymorphic bands. With the exception of *Tvv1*Fa-Ms14 and *Tvv1*Fa-Ms15, all of the primer combinations produced unique bands, resulting in one IRAP, 10 REMAPs, 9 SSAPs and 15 AFLPs (Table 4).

The PIC averages were calculated for each marker system (Table 4), and the highest value (0.2996) was observed for AFLP, although the individual PIC values for some primer combinations were higher. The highest SI (0.5473) was observed in the SSAP primer combination *Tvv1*Fa-M36, and the highest SI average (0.4603) was observed for SSAP (Table 4). The MI reached its highest values for AFLP, with an average of 22.7567, followed by SSAP (Table 4), and these two systems detected a high number of loci. For the REMAP primer combinations, *Tvv1*Fa-Ms15 and *Tvv1*Fa-UBC890-2 represented the lowest and the highest MI values, respectively (Table 4).

IRAP, REMAP, SSAP and AFLP allowed for the discrimination of 37, 40, 40 and 39 different genotypes, respectively (Fig. 2). Of the 41 cultivars that were studied, the Pinot Noir/Pinot Blanc and Moscatel Galego Roxo/Moscatel Galego Branco pairs are considered to be berry skin-colour mutants. The nuclear microsatellites amplification that was performed in previous studies failed to distinguish these pairs of cultivars [31]. In this study, both REMAP and SSAP amplifications could distinguish between the Pinot pair, but not between the Moscatel pair (Fig. 2). The REMAP *Gret1*Fa-Ms8 and *Tvv1*Fa-MS15 primer combinations and the SSAP *Tvv1*Fa-Ms36 primer combinations amplified five different fragments in both Pinots.

The IRAP dendrogram was obtained from 16 polymorphic fragments from the *Tvv1*Fa amplification and failed to distinguish between Malvasia Fina/Aragonez, Touriga Franca/Trajadura, the two Pinot and the two Moscatel cultivars. The REMAP, SSAP and AFLP dendrograms show that several groups of cultivars are clustered in a similar manner, e.g., Tinta Barroca, Touriga Franca, Touriga Nacional and Barca; Baga, Malvasia Fina and Bical; Loureiro and Sousão Galego; and Melhorio and Amaral (Fig. 2). The bootstrap values were higher than 40% in ten or more cases in all of the dendrograms with the exception of IRAP (Fig. 2). The correlation coefficients—estimated using the Mantel matrix correspondence test on the genetic similarity matrices—and cophenetic matrices—generated using the IRAP, REMAP, SSAP and AFLP techniques—were calculated. No significant correlations were observed, but the highest values were found between the AFLP and REMAP data, followed by the SSAP and AFLP data, and the lowest values were those involving IRAP (data not shown).

Clonal Identification

To assess the authenticity of the clones, a true-to-type confirmation of the studied clones to each cultivar (Table 1) was carried out using nuclear microsatellite analysis. Clones of Alvarinho, Arinto, Loureiro, Moscatel Galego Branco, Trajadura and Vinhão were analysed using the same molecular markers and primer combinations as the cultivars (see Table 4). The presence or the absence of 4 REMAP, 48 AFLP, and 37 SSAP fragments indicate clones of the above-mentioned varieties (Table 5; Fig. 3). The IRAP amplifications did not detect polymorphism between clones. The four REMAP markers allowed for the distinction of three clones, Moscatel Galego Branco MG0420, Trajadura TJ1207 and Vinhão VN0530. AFLP and SSAP polymorphic markers identified 15 and 9 clones, respectively, in all the six of the analysed cultivars. The clones that showed the highest number of specific fragments (by their presence or their absence) were Trajadura TJ1020 and Loureiro L0959, with 31 and 16 fragments found, respectively. Of the 37 analysed clones, 20 (54.1%) were identified by specific markers.

Discussion

Cultivar Polymorphisms and Genetic Relationships

Extensive studies using nuclear microsatellites have shown that there is a large amount of genetic polymorphism between Portuguese cultivars [31, 45–49]. The nuclear microsatellite molecular marker method is the approach

Table 4 Results of the observed genetic diversity in the 41 studied cultivars

Technique	Primer combination	TB	PB	UB	SI	MI	PIC
IRAP							
	*Tvv1*Fa	16	16	1	0.4864	3.7720	0.2902
REMAP							
	*Gret1*Fa-Ms8	22	21	2	0.3448	6.4961	0.3093
	*Gret1*Fc-Ms10	17	16	4	0.3751	4.2353	0.2647
	*Tvv1*Fa-Ms13	17	16	1	0.4457	4.5068	0.2817
	*Tvv1*Fa-Ms14	17	17	0	0.4287	4.0984	0.2732
	*Tvv1*Fa-Ms15	12	10	0	0.5119	1.9855	0.2836
	*Tvv1*Fa-UBC890-2	30	28	3	0.4506	7.8859	0.3033
Total		115	109	10			
Average		19.17	18.2	1.67	0.4261	4.8680	0.2860
AFLP							
	E34-M36	113	90	4	0.4343	23.6641	0.2958
	E34-M40	103	77	11	0.4248	21.8492	0.3034
Total		216	167	15			
Average		108	83.5	7.5	0.4296	22.7567	0.2996
SSAP							
	*Gret1*Fa-M36	87	78	8	0.3733	18.3031	0.2613
	*Tvv1*Fa-M36	83	59	1	0.5473	14.6640	0.3120
Total		170	137	9			
Average		85	68.5	4.5	0.4603	16.4836	0.2867

TB Total of bands, *PB* polymorphic bands, *UB* unique bands, *SI* Shannon's index, *MI* marker index, *PIC* polymorphic information content

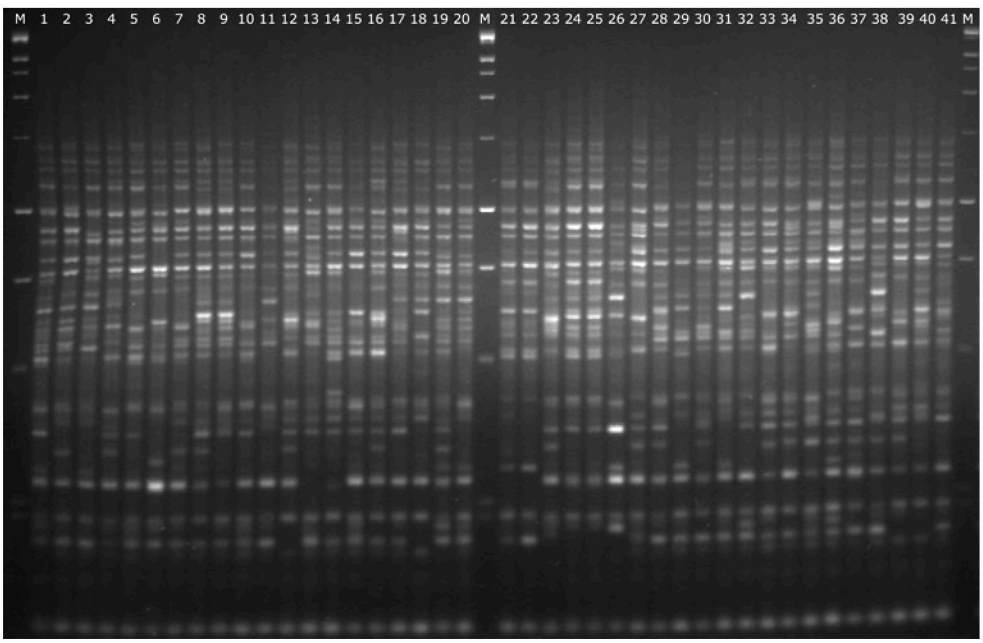


Fig. 1 REMAP electrophoretic pattern generated by the *Tvv1*Fa-UBC890-2 primer combination in 41 grapevine cultivars. 1 Alfrocheiro; 2 Alvarinho; 3 Baga; 4 Arinto do Interior; 5 Arinto; 6 Avesso; 7 Azal; 8 Melhorio; 9 Amaral; 10 Batoca; 11 Borraçal; 12 Alvarelhão; 13 Padeiro; 14 Chasselas; 15 Espadeiro; 16 Gouveio; 17 Lameiro; 18 Loureiro; 19 Malvasia Fina; 20 Bical; M 1 kb DNA Ladder (Promega); 21 Moscatel Galego Branco; 22 Moscatel Galego Roxo; 23 Pedral; 24 Pinot Blanc; 25 Pinot Noir; 26 Rabigato; 27 Rabo de Ovelha; 28 Sousão; 29 Sousão Galego; 30 Vinhão; 31 Tália; 32 Trincadeira; 33 Tinta Barroca; 34 Tinta Carvalha; 35 Barca; 36 Tinta Francisca; 37 Aragonez; 38 Tinto Cão; 39 Touriga Franca; 40 Touriga Nacional; 41 Trajadura; M 1 kb DNA Ladder (Promega)

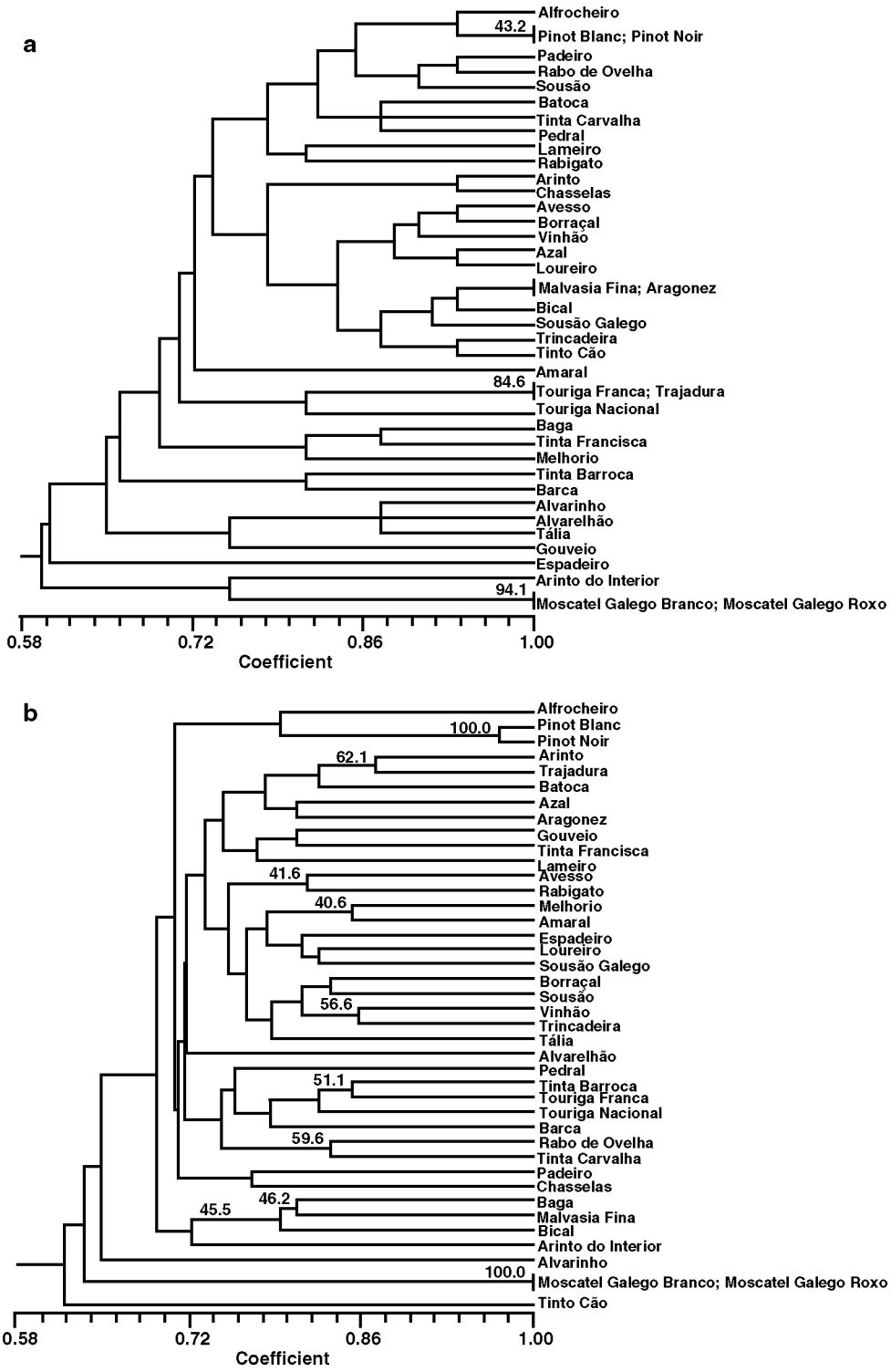


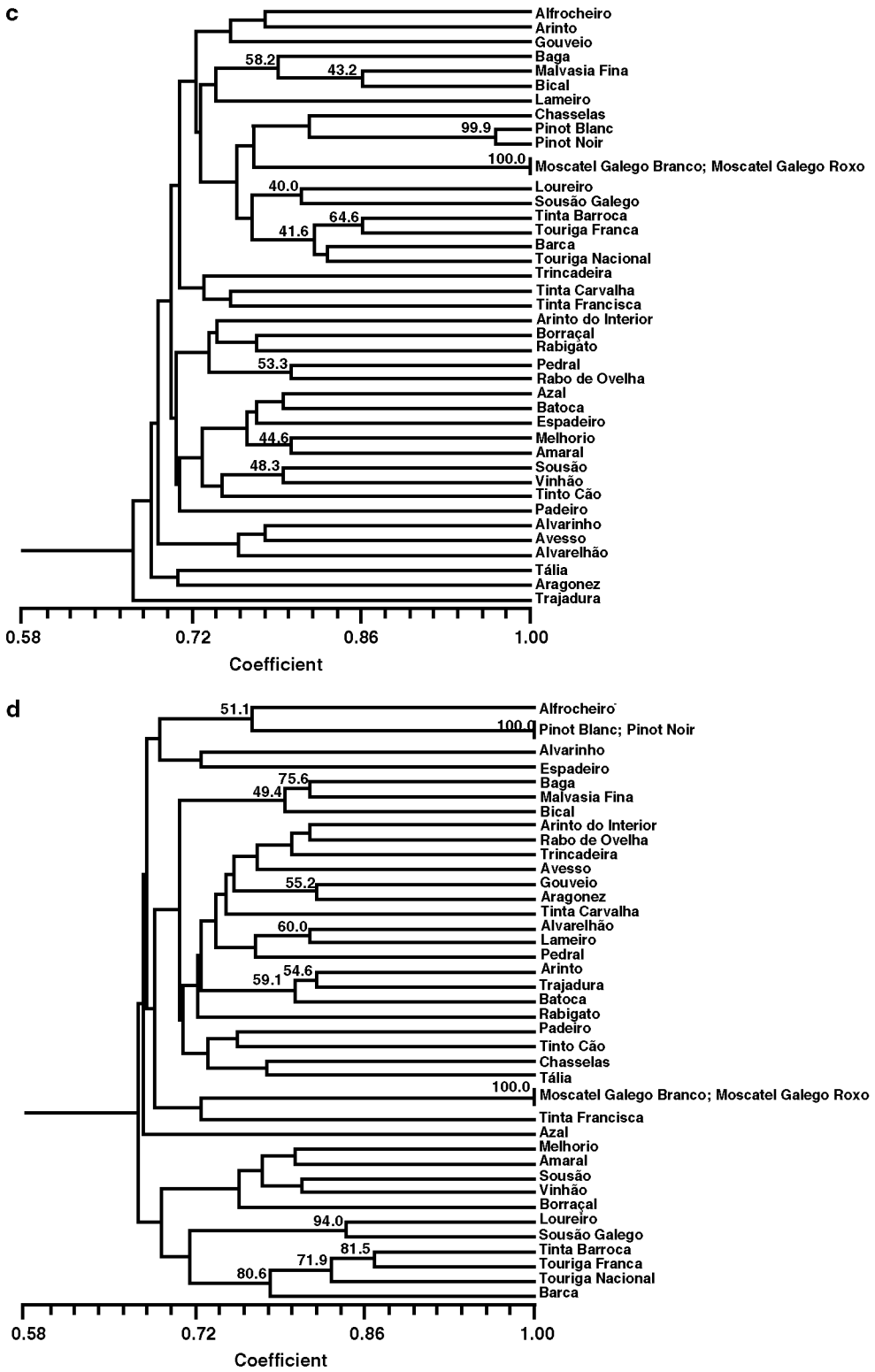
Fig. 2 Dendrograms of 41 cultivars obtained using UPGMA cluster analysis of IRAP (**a**), REMAP (**b**), SSAP (**c**) and AFLP (**d**) marker data. Bootstrap values higher than 40% are indicated at nodes

that is most widely used for the identification of grapevine material.

Although microsatellite markers have been extremely useful in identifying grapevine cultivars, they are monolocus and are not useful for the calculation of the genetic distances between cultivars [29].

Molecular marker techniques, based on retrotransposons; IRAP, REMAP and SSAP; and AFLP, simultaneously amplify more than one locus, and the resulting patterns are sufficiently complex to calculate genetic distances between cultivars and, therefore, to determine evolutionary inferences.

Fig. 2 continued



In this study, of the grapevine retrotransposons (*Tvv1*, *Gret1* and *Vine-1)* that were considered for IRAP, the *Tvv1*Fa primer was the only one that amplified bands that were suitable for analysis, and the *Vine-1* primers used for the REMAP amplicons resulted in mostly ISSR fragments. For four Portuguese cultivars, two of which, Loureiro and Touriga Nacional, are included in this study, only a few IRAP and REMAP bands using *Gret1* sequences were

Table 5 Identification of clones of Alvarinho (AL), Arinto (AR), Loureiro (L), Moscatel Galego Branco (MG), Trajadura (TJ) and Vinhão (VN) by the presence (+) or absence (−) of specific markers using different techniques

Technique	Clones	Primer combination and fragment sizes (bp)
REMAP	MG 0420	*Gret1*Fc-Ms10: 1625(+)
		*Tvv1*Fa-UBC890-2: 345(+)
	TJ1207	*Gret1*Fc-Ms10: 1875(+)
	VN0530	*Gret1*Fc-Ms10: 1625(+)
AFLP	AL1025	E34-M36: 411(−)
	AL1050	E34-M36: 427(+)
	AR2404	E34-M36: 319(−)
	AR2425	E34-M40: 419(+)
	AR3502	E34-M36: 318(−)
		E34-M40: 144(−); 231(+)
	AR8204	E34-M40: 419(+)
	L0734	E34-M40: 233(−); 273(−); 274(−); 389(−)
	L0959	E34-M36: 122(+); 593(−)
		E34-M40: 419(+); 447(+); 449(−); 615(+)
	MG0501	E34-M36: 394(+)
	MG1118	E34-M36: 345(+)
	MG1306	E34-M36: 344(+)
	TJ1020	E34-M36: 128(−); 134(−); 142(−); 156(−); 159(−); 167(−); 214(−); 318(−); 383(−); 404(+); 411(−); 547(+)
		E34-M40: 100(−); 118(−); 132(−); 136(−); 144(−); 281(−); 305(+); 396(−); 413(−); 519(−)
	TJ1103	E34-M36: 593(−)
		E34-M40: 418(−)
	TJ1207	E34-M40: 268(−)
	VN0340	E34-M36: 114(+)
		E34-M40: 250(−)
SSAP	AL1025	*Tvv1*Fa-M36: 300(−); 390(−)
	AR2404	*Gret1*Fa-M36: 349(−)
	L0959	*Tvv1*Fa-M36: 105(+); 106(−); 252(+); 317(−); 328(−); 410(+)
		*Gret1*Fa-M36: 220(+); 322(+); 395(−); 407(−)
	MG0207	*Gret1*Fa-M36: 236(+)
	MG0420	*Tvv1*Fa-M36: 393(−)
		*Gret1*Fa-M36: 449(−); 457(−)
	TJ1020	*Tvv1*Fa-M36: 107(−); 358(−); 385(−); 450(−); 486(−); 576(−)
		*Gret1*Fa-M36: 148(−); 177(−); 276(+)
	TJ1126	*Tvv1*Fa-M36: 328(−)
	VN0340	*Tvv1*Fa-M36: 104(+); 105(−); 106(+); 252(−); 410(−)
		*Gret1*Fa-M36: 210(−); 212(−); 342(−); 343(−)
	VN0514	*Gret1*Fa-M36: 365(−)

detected [29]: those authors suggest, as an explanation for the results, the possibility that retrotransposons are clustered in a nested fashion and are inserted in tandem or that the distance between retrotransposon copies in the clusters or between retrotransposon and the microsatellite loci may be too great to be amplified using conventional PCR methods. These possibilities could also explain the weak amplification results that were observed in this study for IRAP using *Gret1* and *Vine-1* primers and the prevalence of ISSR fragments in REMAP using *Vine-1* sequences.

REMAP, SSAP and AFLP demonstrated a high capacity for distinguishing the grapevine cultivars. REMAPs have the disadvantage of requiring the amplification of more primer combinations to obtain as high a number of fragments as seen in SSAP and AFLP; however, these latter two techniques are more complex and include endonuclease digestion, the ligation of adaptors and preselective and selective amplifications. The REMAP and SSAP markers obtained in this study, which are based on *Tvv1* and *Gret1* retrotransposon sequences, were successful in

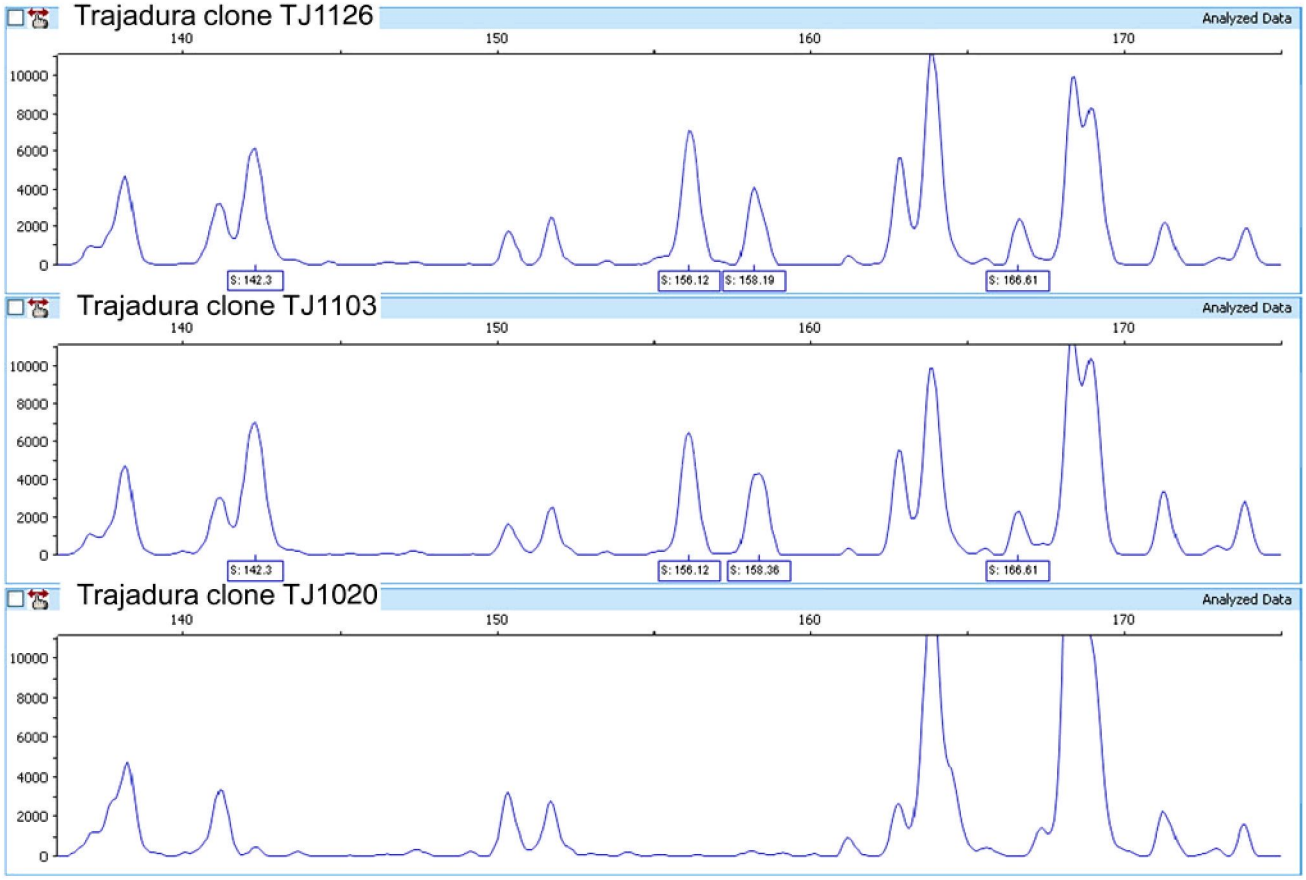


Fig. 3 Region of the densitograms obtained for three of the five Trajadura clones analysed (TJ1020, TJ1103 and TJ1126) using the AFLP combination of primers E34-M36, where four polymorphisms, by absence, are observed in the TJ1020 clone (approximately 142, 156, 158 and 167 bp)

discriminating the two Pinot mutants but not the two Moscatel Galego mutants. Taking into consideration that *Gret1* was isolated as a retrotransposon-induced mutation of *VvmybA1*, a gene that affects grape skin colour [13], it was expected that the *Gret1* sequence would result in the amplification of some different retrotransposon-based markers in grape colour mutants. However, the amplified fragments that were observed in Pinot Noir and Pinot Blanc were based on LTR primers of *Gret1* and *Tvv1* and were detected in other black or white cultivars.

The dendrograms obtained with AFLP and SSAP may be more reliable because they are supported by higher bootstrap values. Consequently, these two methodologies are more appropriate for the analysis of genetic distances between cultivars and for the calculation of evolutionary inferences without the necessity for cultivar identification and pedigree.

Clonal Variability

The results of the application of the molecular markers included in this study for the detection of clonal polymorphism in grapevines depend upon the intravarietal variability of the studied cultivars. Stajner et al. [4] reported the successful identification of 18 Malbec and 13 Syrah clones using an average of 30 and 34 AFLP and SSAP polymorphic bands, respectively. Using a modified transposon display approach that is based on the original SSAP procedure but that uses primers that are universal for several types of plant retrotransposons, Wegscheider et al. [50] observed a 4.8% clonal variation that differentiated four out of five Pinot noir clones. Recently, using AFLP markers, Anhalt et al. [51] reported the analysis of 86 Riesling clones with a considerable number of polymorphic bands (135 out of 305), and noted a variability between DNA replicates of 0%–0.7% and a variability of 0% in technical replicates. The analysis of the Italian Sangiovese cultivar clones carried out by Filippetti et al. [52] using AFLP discriminated only three polymorphic clones out of 26, and D'Onofrio et al. [26] reported no stable polymorphisms in some clones of the same cultivar that were analysed using AFLP and SSAP. In the important Portuguese cultivar Touriga Nacional, the analysis of the *Gret1* polymorphism using IRAP and REMAP failed to detect variability in four clones [29].

The antiquity of the cultivar and the respective clones [4, 53], the set of markers chosen for DNA amplification [1], and the number and geographical areas of distribution of the clones that were analysed, are of great importance for the search of intracultivar variability.

The two certified clones that showed the highest degree of variability, Loureiro L0959 (84ISA) and Trajadura TJ1020 (87ISA), belong to two old autochthonous cultivars from the northwest Iberian Peninsula. These cultivars correspond to Loureiro and Treixadura cultivars from Galicia in Spain [54]. Five Spanish clones from each of these cultivars have been previously analysed using SSR and standard and modified AFLP methodology, and no polymorphism was detected [55].

In conclusion, this work shows that the IRAP marker system that is based on *Tvv1*, *Gret1* and *Vine-1* retrotransposons is a weak technique for the identification of cultivars and is inadequate for the detection of intracultivar variability. REMAP was a more straightforward methodology, with a high capacity for distinguishing cultivars and a lesser capacity to distinguish clones. The application of these methodologies, along with other retrotransposon classes that have been discovered through the generation of new information from the grapevine genome, may permit the detection of high levels of variability. An increase in the number of primers used would also provide more discriminating power to study closely related genotypes and to perform analysis within cultivars. SSAP and AFLP were shown to be the most polymorphic among the marker systems that were tested, and they appear to be the most appropriate for clone identification. This study provides an important molecular contribution to the study of clonal variability and identification. The activities of retrotransposons exhibit a close relationship with stresses, such as high temperatures [25]. In a climate-change scenario, grapevine cultivars that demonstrate a special aptitude for resisting high temperatures and exhibit late maturation are of great importance [56]. Retrotransposon-based molecular markers are extremely helpful for identifying these particular cultivars.

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